ANTISENSE OLIGO 3'-poly-L-lysine, 5' end target

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Inhibition of expression of SV40 virus large T-antigen by antisense oligodeoxyribonucleotides*

P. Westermann, B. Gross, and G. Hoinkis

Zentralinstitut für Molekularbiologie, Akademie der Wissenschaften der DDR, 1115 Berlin-Buch, DDR

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Summary

Expression of large *T*-antigen in COS cells can be inhibited by treatment of cell monolayers with oligodeoxyribonucleotides complementary to large *T* mRNA, which were covalently linked to poly-L-lysine. Strongest inhibition was observed with conjugates of oligodeoxynucleotides that hybridize to the sequence immediately 3' to the cap structure of the mRNA. Treatment of SV40 virus-infected CV-1 cells with the same conjugates reduces the virus-induced large *T*-antigen expression by more than 80 %.

Introduction

Inhibition of virus replication has been observed in cells transfected with plasmids containing virus gene sequences in "antisense" orientation [1, 2]. Since a prerequisite for a successful medical application of this procedure is a complete transfection of all cells, alternative methods have been searched for. The direct way of treating cells with chemically synthesized oligodeoxyribonucleotides complementary to different parts of the virus genome was used for the inhibition of Rous sarcoma virus replication [3], and for inhibition of the expression of HIV-1 reverse transcriptase and of virus proteins [4, 5]. In order to increase the uptake of oligodeoxyribonucleotides by cultured cells, calcium phosphate precipitation was applied [6]. Other approaches to penetrate the cell membrane barrier are based on oligodeoxyribonucleotides chemically modified with methylphosphonate groups [7, 8] or with 3' or 5'-terminal aminoacridine groups [9]. By introduction of 5'-terminal 4-(N-2-chloroethyl-N-methylamino)benzyl-phosphamide groups covalent linkages between the oligodeoxynucleotide and the target nucleic acid can be formed [10]. A significant inhibition of virus replication in cell culture is not observed at concentrations of oligodeoxynucleotides less than 20 μM, and in most cases 100 μM of the oligodeoxynucleotide have to be applied. An increase in the effectivity of an "antisense" oligodeoxynucleotide is observed by covalent attachment of poly-L-lysine [11, 12], a method originally developed to increase the uptake of enzymes by cultured cells [13]. In this case, an inhibitory concentration of 100 nM was sufficient to suppress in vitro the replication of vesicular stomatitis virus [12].

In order to compare the inhibitory effect of different "antisense" oligodeoxynucleotides conjugated to poly-L-lysine, the expression of SV40 virus large T-antigen was investigated. Oligodeoxynucleotides complementary to different parts of the 5'-untranslated sequence of large T mRNA were synthesized and covalently linked to poly-L-lysine. Conjugates containing an oligodeoxynucleotide complementary to the sequence 3' to the cap site of large T mRNA possess the strongest inhibitory effect on large T-antigen expression in COS cells and block also virus replication in CV-1 cells.

* Dedicated to Prof. Dr. H. Bielka on the occasion of his 60th birthday

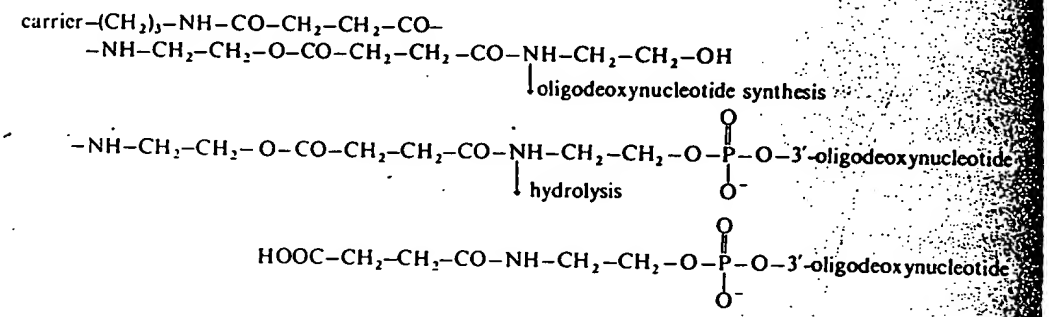
Materials and methods

Cells

CV-1 cells and COS cells [14] were cultivated in Eagle-MEM medium containing 10% calf serum.

Synthesis of oligodeoxyribonucleotide poly-L-lysine conjugates

Oligodeoxynucleotides complementary different regions of large *T* mRNA (compare Fig. 1) were synthesized with a DNA synthesizer 380B (Applied Biosystems, USA) using glass beads (Serva glass, pore size 100 nm) derivatized with a modified spacer that can be hydrolysed after completion of synthesis resulting in the formation of a 3'-carboxy group [14]:



The 3'-carboxy-modified oligodeoxynucleotides were coupled in a molar ratio of 1:1 to poly-L-lysines of molecular weights of 3700, 6000 and 50000 by addition of a 10-fold molar excess of 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide-hydrochloride in a buffer containing 2.5 M sodium chloride and 0.1 M sodium morpholinoethansulfonate, *p*H 5.0. The conjugates were purified by Sephadex G25 gel chromatography and stored in 1.4 M sodium chloride, 20 mM Tris-hydrochloride, *p*H 7.5 (buffer A).

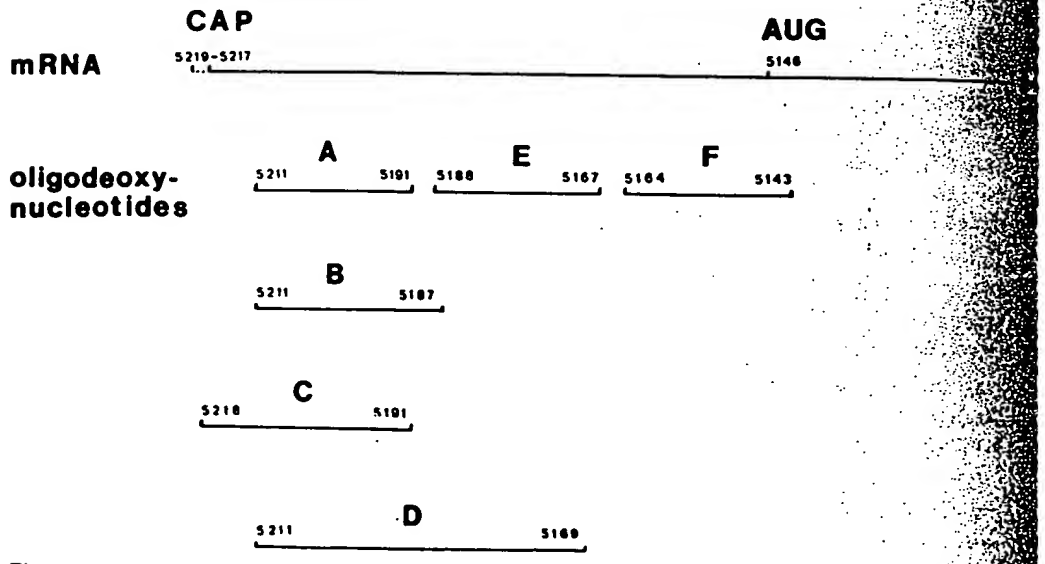


Fig. 1. Arrangement of oligodeoxyribonucleotides A—F within the 5'-terminal sequence of the mRNA of SV40 large T-antigen

Inhibition of large T-antigen expression in COS cells

To 10^5 COS cells in multidish cell culture plates (24 wells) were added 0.2 ml MEM medium with a reduced methionine concentration (9 mg/ml), supplemented with 10% calf serum. The corresponding oligodeoxynucleotide poly-L-lysine conjugates in buffer A were added, and the sodium chloride concentration was adjusted to 0.14 M. After 4 h incubation at 37 °C in 5% atmosphere 5 µCi [^{35}S]methionine were added and the incubation was continued for 16 h.

Thereafter cells were lysed in 0.1 ml buffer B containing 0.14 M sodium chloride, 0.003 M potassium chloride, 0.02 M Hepes, *p*H 7.5, 0.1 mM phenylmethylsulfonylfluoride and 0.5% Triton X-100. After centrifugation for 5 min at 12000 × *g* the supernatant was mixed with 10 µl hamster anti *T* serum and incubated for 4 h at 22 °C. After addition of a suspension of formaldehyde-fixed Staphylococcus aureus cells the incubation was continued for additional 4 h. The resulting pellet was washed three times with buffer B and thereafter extracted with SDS sample buffer (1% sodium dodecylsulfate, 0.1 M Tris-HCl, *p*H 7.5, 1% mercaptoethanol, 20% glycerol).

The proteins were separated according to LAEMMLI [15] using a 7.5—12% gradient gel. After fixation of the gel in a mixture of methanol, acetic acid, and water (5:1:4), incubation in methanol and soaking in Amplify (Amersham, UK) the gel was dried between cellulose acetate sheets. Fluorography using Kodak X-Omat film was for 64 h at −70 °C.

Inhibition of SV40 virus-mediated large T-antigen expression in CV-1 cells

$2—3 \cdot 10^4$ CV-1 cells per well were cultivated in 0.5 ml MEM medium with 10% calf serum. The oligodeoxyribonucleotide poly-L-lysine conjugates in buffer A were added after diluting with 9 volumes MEM medium produced without sodium chloride. Infection with SV40 virus was done by addition of 100 µl SV40 virus-containing medium either 4 or 16 h before addition of the conjugate, or at the same time or 4 h after addition of the conjugate. The medium was changed after 24 h and a part of the samples was supplied with a second portion of the conjugate. Using some of these samples a third group received an additional treatment with conjugate at the third day. At the 4th day cells were trypsinized and seeded on cover slips. On the 6th and 7th day samples were taken, rinsed with buffer C (0.14 M sodium chloride, 0.02 M Hepes, *p*H 7.5), and fixed for 15 min in acetone. Expression of large *T*-antigen was demonstrated by addition of hamster anti *T* serum diluted 1:4 with buffer C, incubation for 30 min at 37 °C, rinsing with buffer C, and incubation with FITC-labelled anti hamster IgG from rabbit (diluted 1:40 with buffer C) for 30 min at 37 °C.

Fluorescent nuclei (Fig. 2) were counted and related to the total number of cells. The average expression of large T-antigen was measured as described before for COS cells.

Results

Inhibition of large T-antigen expression in COS cells

COS cells express large T-antigen that can be visualized by two-dimensional electrophoresis [16] and is precipitated by anti T serum [17]. For studying "antisense" inhibition the effect exerted by the oligodeoxyribonucleotides had to be measured over several hours. Therefore cells were labelled 4 h after administration of the oligodeoxynucleotide conjugates for 16 h with [^{35}S]methionine in the presence of low concentration of unlabelled methionine in the medium. Large T-antigen was then immunoprecipitated from cell lysate and identified by polyacrylamide gel electrophoresis and fluorography.

Treatment of COS cells with 80 µg conjugate of oligodeoxynucleotide A and poly-L-lysine of molecular weight 3700 or 6000 reduces the labelling of large T-antigen by 52% or 74%, respectively (see Tab. 1). Application of 40 µg/ml conjugate diminishes the effect; below 20 µg/ml no inhibition could be measured (data not shown).

Table 1
Inhibition of large T-antigen expression in COS cells by antisense oligodeoxynucleotides[1]

Oligodeoxynucleotide[2]	conjugated to poly-L-lysine (molecular weight)	Inhibition of expression
A	3700	52%[3]
	6000	74%[3]
B	3700	no inhibition[4]
C	3700	no inhibition[4]
	6000	no inhibition[4]
D	3700	no inhibition[4]
	6000	25%
E	6000	no inhibition[4]
F	6000	56%[3]

[1] The percentage of inhibition in relation to untreated controls is given as mean value of three experiments.
[2] The concentration of oligodeoxynucleotide (in form of the conjugate) is 80 µg/ml medium.
[3] Mean square error 10%.
[4] Inhibition, if any, is smaller than 10%.

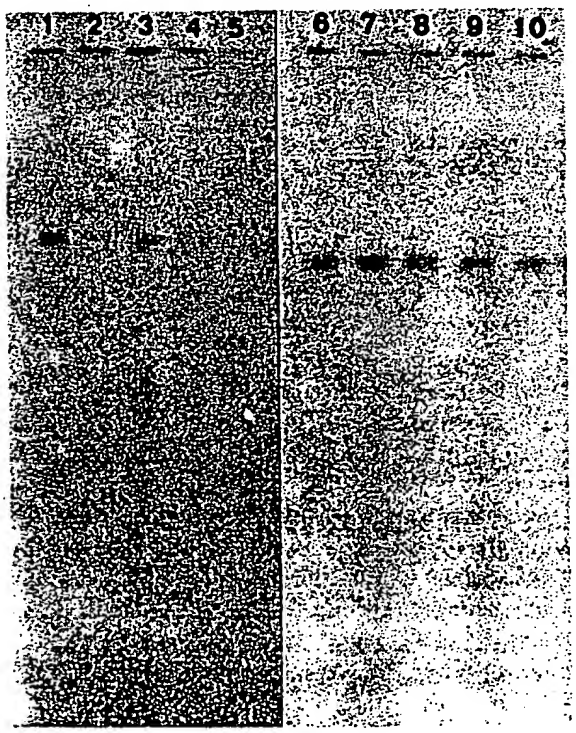


Fig. 2. Inhibition of large T-antigen expression by oligodeoxyribonucleotide poly-L-lysine conjugates in COS cells.
1 and 6: untreated controls; 2—5: COS cells treated with 5, 10, 20, 40 or 80 µg oligodeoxynucleotide A conjugated to poly-L-lysine of 6 kDa; 7—10: COS cells treated with 5, 10, 20, 40 or 80 µg oligodeoxynucleotide F conjugated to poly-L-lysine of 6 kDa

Increasing the length of the oligodeoxynucleotide, e.g. in case of B, C, and D, reduces strongly the inhibition. It is also evident from experiments with conjugates of A and D that poly-L-lysine of a molecular weight 6000 forms more potent inhibitors than poly-L-lysine of molecular weight 3700. A further increase in molecular weight by using poly-L-lysine of 50 kDa results in the formation of toxic conjugates. Comparing the effects of A, E, and F conjugated to poly-L-lysine of molecular weight 6000 shows that the 5'- and 3'-terminal parts of the 5'-untranslated region of large T mRNA can be used for "antisense" inhibition by conjugate A and F, respectively (see Fig. 2), whereas in the case of conjugate E, containing an oligodeoxynucleotide complementary to the middle part of the 5'-region, no inhibition was observed.

Inhibition of large T-antigen expression in SV40 virus-infected cells

SV40 virus infection of in vitro cultivated CV-1 cells takes 6 to 7 days until large T-antigen expression can be monitored by the immunofluorescence technique described under "Material and methods". This method allows to measure the rate of large T-antigen expression, which is a necessary prerequisite of SV40 virus replication. The results obtained point to a reduction of the percentage of large T-antigen expressing cells and not to a partial reduction of expression in all cells.

Oligodeoxyribonucleotides as such possess no inhibitory activity in concentrations up to 10 µg per ml medium, and also repeated addition during the first, second, and third day of infection does not reduce the expression of large T-antigen neither in infected CV-1 cells nor

Table 2
Inhibition of large T-antigen expression in SV40 virus-infected CV-1 cells by antisense oligodeoxynucleotides[1]

Oligodeoxynucleotide[2]	conjugated to poly-L-lysine (molecular weight)	Treatment of cells with conjugate					
		on day 1		on day 1 and 2		on day 1, 2 and 3	
		Analysis on day 6	day 7	day 6	day 7	day 6	day 7
A	3700	51%	/ 32%	82%	/ 75%	85%	/ 80%
	6000	55%	/ 41%	79%	/ 63%	86%	/ 72%
B	3700	52%	/ 49%	75%	/ 70%	62%	/ 73%
C	6000	85%	/ 69%	88%	/ 72%	80%	/ 85%
D	3700	no inhibition[3]					
	6000	21%	/ 12%	22%	/ 25%	26%	/ 32%
F	6000	35%	/ 41%	n.d.		n.d.	

[1] The percentage of inhibition in relation to untreated controls is given as mean value of three experiments.
[2] The concentration of oligodeoxynucleotides is 10 µg/ml medium.
[3] Inhibition, if any, is smaller than 10%.
n.d., not determined

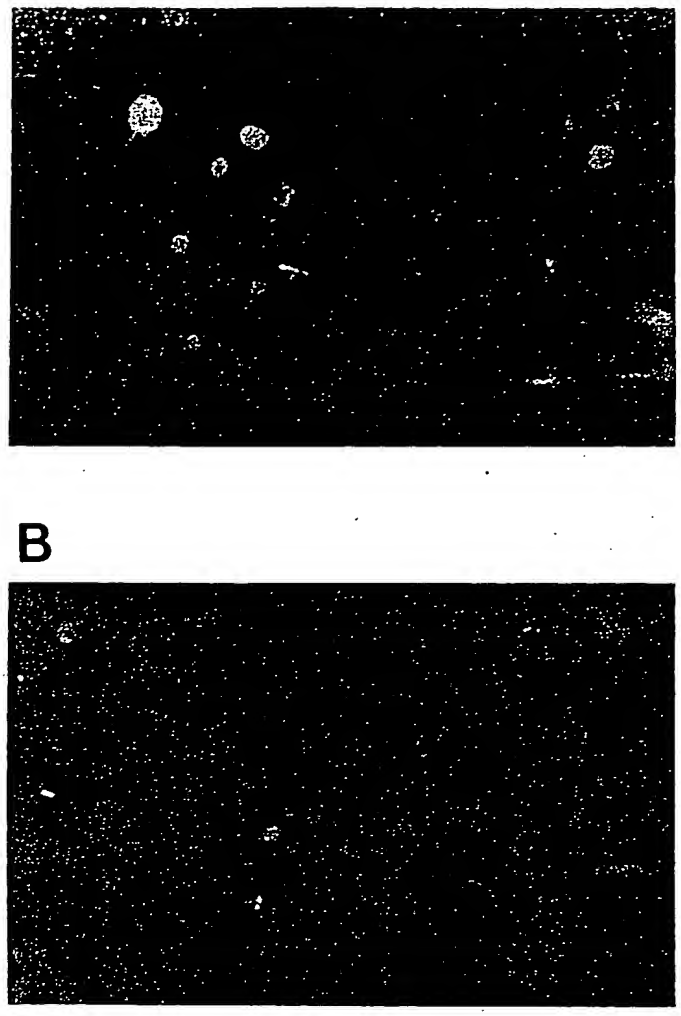


Fig. 3. Inhibition of large T-antigen expression in SV40 virus-infected CV1 cells. A) untreated control analyzed on the 6th day; B) cells treated with 10 µg/ml conjugate of oligodeoxyribonucleotide A and poly-L-lysine of 6 kDa 4 h after infection with SV40 virus

in COS cells. Experiments with ^{32}P-labelled oligodeoxynucleotides proved a rapid degradation within the cells in less than 1 h (data not shown).

Treatment of SV40 virus-infected cells with covalent conjugates of oligodeoxynucleotides and poly-L-lysine inhibits the expression of large T-antigen in analogy to the experiments performed with COS cells. In order to study the kinetics of inhibition, oligodeoxynucleotide poly-L-lysine conjugates were added at different times with respect to infection by SV40 virus. Addition of conjugates either 4 h before infection, at the same time as SV40 virus or, respectively, 4 and 16 h after infection causes no differences in the extent of inhibition. On the other hand, a repeated addition of the conjugate during the first, second and third day of infection increases the inhibitory activity (see Tab. 2).

The expression of large T-antigen is decreased by conjugates of oligodeoxynucleotide A and poly-L-lysine in the range between 51% to 86% on the 6th day (see Fig. 3), and between 32% and 80% on the 7th day depending on the duration of treatment. The results also demonstrate that the inhibition decreases with increasing time between treatment and analysis. Prolonged incubation of cell cultures for up to 12 days without additional conjugate treatment further increases the expression of large T-antigen (data not shown).

Comparison of the effects of conjugates of oligodeoxyribonucleotides A, B, C, and D shows a decrease of inhibition with increasing chain length. In experiments with oligodeoxyribonucleotide D, a 43-mer, it is also evident that conjugation with larger poly-L-lysines results in more potent inhibitors.

Oligodeoxyribonucleotide F, complementary to a region encompassing the AUG codon and the 19 5'-joined nucleotides, shows in SV40 virus-infected CV-1 cells a medium inhibition.

Discussion

Specific inhibition of virus replication or expression of virus proteins by oligodeoxyribonucleotides complementary to 3'-LTR sequences [3] or to primer and splice sites [4, 5] were observed for retroviruses. SV40 virus replicon function was inhibited by antisense RNA directed against the total 5'-untranslated region of large T mRNA encompassing nucleotides 5237—5092 [21]. Therefore, oligodeoxynucleotides complementary to parts of the 5' untranslated sequence were synthesized and added to the medium that was used for cultivation of COS cells. In our experiments, large T-antigen expression in COS cells or SV40 virus replication in CV-1 cells was not inhibited by these oligodeoxynucleotides. Also noncovalent complexes between these oligodeoxyribonucleotides and poly-L-lysine, histone H1, HMG1 protein or DEAE dextran 500 exert no inhibitory activity. As one exception, an inhibition of virus replication was observed by cotransfection of SV40 DNA and oligodeoxynucleotide F using the calcium phosphate precipitation technique in analogy to [6]. The lack of inhibitory activity of the oligodeoxynucleotides may depend in these cells on the low metabolic stability characterized by a half life of 5'-labelled oligodeoxynucleotides of less than 1 h (data not shown). In experiments with HL60 cells, successful inhibition of c-myc expression by a complementary oligodeoxynucleotide was connected with a half life of the oligodeoxynucleotide between 24 and 48 h [18].

In contrast to the results obtained with oligodeoxynucleotides or their complexes with positively charged polymers, covalent conjugates between complementary oligodeoxynucleotides and poly-L-lysines are potent inhibitors of large T mRNA translation. A comparison of poly-L-lysines with average molecular weights of 6000 and 3700 shows that poly-L-lysine with the molecular weight of 6000 yields the stronger inhibitory conjugates. The difference is even more pronounced by application of larger oligodeoxynucleotides. The increasing effect with increasing number of positive charges of the polylysines (18 or 29, respectively) and the decrease in inhibition with increasing number of negative charges of the oligonucleotide (21, 22, 25, 28, and 43) leads to the conclusion that the total charge of inhibitory conjugates should be positive or only weakly negative. The positive charge seems to be necessary for binding of macromolecules to the cell membrane in general and for endocytosis, as it was also demonstrated for proteins [13].

Comparing the parts of the 5'-untranslated sequence of large T-antigen mRNA it becomes evident that the 5' third is the best target for "antisense" inhibition of translation. One

reason might be that this sequence is needed for binding of initiation factors during the first steps of protein synthesis initiation [19]. On the other hand, region 5217—5188 shows no contigous sequences larger than 6 nucleotides with complementarity to other parts of the mRNA and possesses therefore a low degree of secondary structure. As a result an unimpaired hybridization with complementary oligodeoxynucleotides can be expected.

The missing inhibitory effect of oligodeoxynucleotide E may depend on hybridization between sequence 5187-5171 and the complementary sequence 4417-4433 in the coding region; both are homologous in 15 out of 17 bases. The resulting double stranded structure is the largest within the large T mRNA and a hybridization energy of —27.9 kcal/mole can be calculated. The distance between both regions is 390 nucleotides after mRNA splicing.

The region complementary to oligodeoxynucleotide F shows at a maximum 6 contiguous bases complementary to other parts of the mRNA that may explain the successful binding of oligodeoxynucleotide F and its inhibitory effect. On the other hand, the region includes the AUG start codon and a putative binding site for 18S rRNA [19]. Two sequences (5159-5155 and 5162-5165) show a good homology to the consensus sequences of the binding site proposed in [19], and the 3′ terminal one could interact with oligodeoxynucleotide F. Both sequences, the AUG codon and the consensus sequence, are crucial sites for protein synthesis initiation, and their hybridization may inhibit large T synthesis. The comparison of the effects in COS and CV-1 cells shows that SV40 virus-mediated large T expression is more sensitive towards "antisense" inhibition. In this case a concentration of about 10 μg per ml oligodeoxynucleotide in the form of a polylysine conjugate is sufficient for an inhibition of up to 80%. In COS cells between 40 to 80 μg per ml medium are needed for a similar suppression of large T synthesis. An explanation could be that COS cells, obtained by transformation of CV-1 cells as a rapidly proliferating cell line, express enzyme(s) with double strand unwinding activity. Similar activities observed in Xenopus oocytes after fertilization were shown to abolish "antisense" inhibition completely [20].

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